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14. ABSTRACT <p>Fibroblast growth factor binding protein (FGF-BP1) is a crucial molecule that acts to chaperone active FGFs to receptors, thus propagating angiogenic signals for the development of new vasculature. We have shown that FGF-BP1 is expressed in head and neck, skin, cervical, and lung squamous cell carcinomas. A second family member, FGF-BP2 has been identified in our lab and is present in mammary tissue. In this grant, we hypothesized that FGF-BP2 acts in a similar pro-angiogenic capacity as FGF-BP1. The aims were 1) to produce recombinant FGF-BP2 and test its effect on signal transduction, and 2) to study the expression of FGF-BP2 during mouse mammary gland development and carcinogenesis. To date, we have isolated human FGF-BP2 cDNA and protein and confirmed its ability to modulate FGF2. However, we have yet to discover the murine homologue to FGF-BP2. We have identified a third family member, FGF-BP3 in human and mouse. Accordingly, we have adjusted our focus to the characterization of FGF-BP3 activity and expression while continuing our search for murine FGF-BP2. Both human and murine FGF-BP3 have been shown to bind FGF2, promote increased proliferation, MAPK activation, and anchorage-independent growth in SW-13 adrenal carcinoma cells, and murine FGF-BP3 is present in high levels at specific time points in embryonic tissue.</p>					
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Introductions

Tumor growth is dependent upon local-acting growth factors to stimulate the infiltration and growth of new blood vessels from surrounding normal tissue into the tumor mass, a process known as angiogenesis [1,2]. An important class of growth factors that positively regulate angiogenesis is the fibroblast growth factor (FGF) family [3]. Two key members of this family, FGF-1 and FGF-2, require a chaperone molecule to release these factors from extracellular storage and present them to target receptors. These chaperone molecules are known as FGF-binding proteins (FGF-BPs). Two members of the FGF-BP family have been identified so far, FGF-BP1 and FGF-BP2 [4,5]. Previous work from our laboratory has shown that FGF-BP1 can positively modulate the biological activity of FGFs, can support tumor growth and angiogenesis in FGF-2 expressing cell lines, is highly expressed in multiple cancers, and can act as an angiogenic switch *in vivo* in colon cancer cells and SCC. [6,7]. Preliminary data from our lab suggests that FGF-BP2 may act in a similar fashion to chaperone FGFs but has shown distinct expression patterns in tumor samples, particular in human breast cancer samples. A crucial aspect to our study was examination of the expression of FGF-BP2 during mouse development and carcinogenesis, but to date, no murine homologue for FGF-BP2 has been found. Interestingly, a third FGF-BP family member, FGF-BP3 has recently been identified in our lab and a murine homologue for FGF-BP3 was also found. In this study, we began to examine the biological effects of FGF-BP2 but having found mouse and human FGF-BP3, we have refocused our study to explore the effects of this new family member.

Body

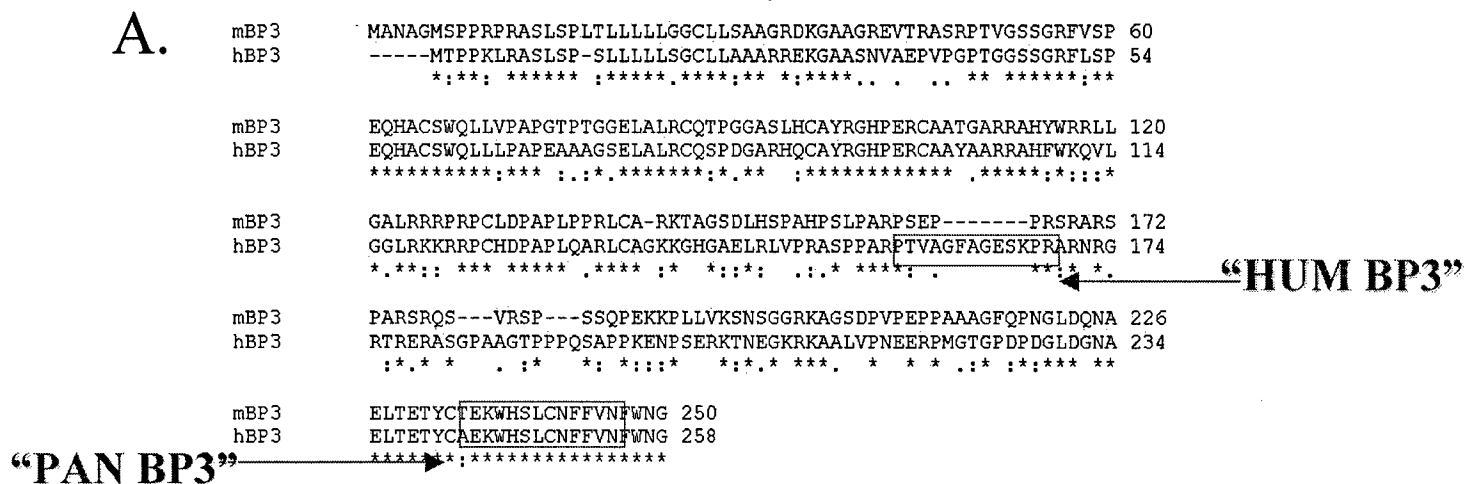
We have previously discovered and investigated a new member of the FGF-BP family, FGF-BP2. In an effort to study the biological significance of human FGF-BP2, our goals outlined in Aim 2 were contingent upon the isolation of a murine homologue to FGF-BP2. However, one candidate murine EST thought to contain FGF-BP2 that was isolated through homology searches using the human and chicken FGF-BP2 gene proved instead to be the murine homologue of just discovered human FGF-BP3 (**Fig 1a**). Accordingly, our research aims have been adjusted to investigate the activity of human and murine FGF-BP3 through many of the processes previously described for FGF-BP2. Although we have progressed with the examination of FGF-BP3, our efforts to identify and isolate a murine homologue to FGF-BP2 have continued, but yielded no discoveries. During the course of our search, we have identified a previously unknown chicken homologue to FGF-BP1 providing us with an updated radial tree depicting the phylogenetic relationship of all known members of the FGF-BP family (**Fig 1b**).

The goals stated in Aim 1 have now been modified to reflect the initial characterization of FGF-BP3. Both human and murine FGF-BP3 have been isolated, amplified, and expressed in a pcDNA3.1 vector construct containing both a V5 and polyhistidine carboxy-terminal tag. Please note that attempts at production of an insect-derived recombinant FGF-BP3 protein will not be made in this project. Rather, the FGF-BP3 proteins produced by SW-13 cells and exported into the cell culture media is able to be isolated via immobilized-metal affinity chromatography (IMAC) in which a special nickel column utilizes the polyhistidine tag on the protein in order for it be isolated from media. In this manner, purified FGF-BP3 is then used in biological and biochemical studies. As had been observed with human FGF-BP1, co-stimulation of human FGF-BP3

with a sup-optimal dose of FGF2 resulted in a synergistic enhancement of FGF2-dependent ERK1/2 phosphorylation in NIH-3T3 murine fibroblasts. Treatment with FGF-BP3 alone has no effect. Furthermore, the presence of a specific inhibitor of FGF Receptor 1 (FGFR1) diminished this enhancement to near baseline levels demonstrating this effect to be regulated by the FGF signaling pathway. (Fig 2) Similar results were observed with murine FGF-BP3 (not shown). As reported previously, SW-13 cells engineered to express human and mouse FGF-BP3 exhibited an increased level of cellular mitogenesis (Fig 3a) and SW-13 cells engineer to express FGF-BP3 showed increased levels over control of anchorage-independent growth on par with levels observed with FGF-BP1 expressing cells as determined through colony formation (Fig 3b). A neutralizing antibody towards FGF2 eliminated both the mitogenic effect and the increased colony formation showing this effect to again be FGF2-dependent. Immunoprecipitation assays suggested specific complex formation between human FGF-BP3, FGFR1, and FGF2. A cell-free binding assay was specifically developed for this project to help elucidate the binding profile of FGF-BP3. Early results show that concentrated conditioned media containing either human or murine FGF-BP3 is able to specifically bind immobilized FGF2 (not shown). Currently, we are undertaking a more detailed analysis of FGF-BP3 binding capacity through the use of the Biacore System for protein interaction which incorporates label-free surface plasmon resonance based technology for studying and quantifying biomolecular interactions in real-time. **Our data indicates that both human and murine FGF-BP3 can significantly enhance FGF-dependent proliferative activity in both fibroblastic- and cancer-derived cell lines. Accordingly, FGF-BP3 may act as an important regulator for the promotion of carcinogenesis in a tissue-specific manner**

The goals of Aim 2 have essentially remained intact but now focus on the expression of FGF-BP3 mRNA during total mouse development. Previous experiments with appropriate controls indicated a ubiquitous rather than specific FGF-BP3 mRNA staining pattern in 11-day old mouse embryo sections. In an attempt to isolate a better time-point to study FGF-BP3 mRNA expression in developing mouse embryos, Northern Blot analysis was undertaken on tissue isolated from various stages of development. Interestingly, our analysis shows a remarkable spike of FGF-BP3 expression at day 10.5 with a subsequent drop-off in expression around day 14.5 (Fig 5). Furthermore, *in situ* hybridization analysis in mouse embryo sections from day 8 through 16 reinforce this finding with earliest staining observed at day 9 and a near complete lack of staining by day 14. Interestingly, analysis of mRNA expression at these time points reveals a ubiquitous staining pattern throughout the entire developing embryo, not clustered mRNA expression in specific developing tissue as expected. To help elucidate potential tissues of maximal FGF-BP3 expression, we then examined mRNA levels in a sample of 12 organs in adult mice using quantitative real time PCR. After screening a pool of male and female adult mice, we were able to identify organs of high relative expression, such as the skin, brain, and eyes as well as regions of extremely low relative levels including the organs associated with the gastro-intestinal tract and sex organs (Fig 6). As some of this data correlates with previous data indicating high levels of FGF-BP3 in human neuroblastoma- and retinoblastoma-derived cell lines as observed with standard RT-PCR, we sought to expand the examination of FGF-BP3 expression via *in situ* hybridization analysis of tissue samples from human normal and cancerous brain tissue. Preliminary data suggests that FGF-BP3 is indeed over-expressed in a variety of human brain-derived cancer tissues including neuroblastoma, astrocytoma, and meningioma. A more

complete survey is underway. Aim II, Series #2 has also been adjusted to allow for the creation of peptide antibodies against regions of both human and mouse FGF-BP3. Specifically, one set of antibodies have been targeted to a 12-mer found only in human FGF-BP3, named "HUM BP3", while a second set of antibodies have been targeted to another 12-mer with 100% homology between both human and mouse FGF-BP3, "PAN BP3" (Fig 1a). Additionally, peptide antibodies have been created against a 12-mer located on the carboxy-terminal end of human FGF-BP2 that will help with our continuing search for its biological activity. Currently, we have only achieved success in the development of a human specific antibody with use in Immunoblot analysis. Attempts to optimize its use in Immunohistochemical analysis have yet to yield any positive results. **Our data indicates that FGF-BP3 expression is highly up-regulated during a specific time frame in embryonic development as witnessed through both Northern blot and *in situ* hybridization analysis. Additionally, FGF-BP3 expression appears concentrated to specific organs in adult mice such as the brain and eye and may play a prominent role in cancer development in these tissues.**



B.

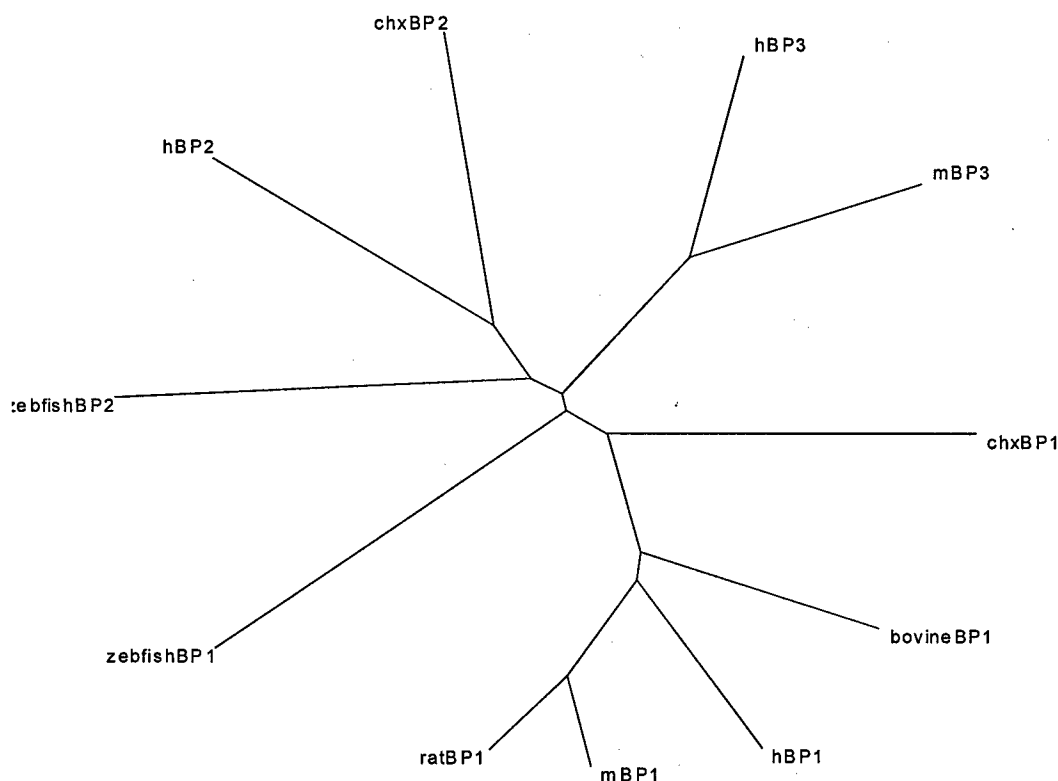


Figure 1: Phylogenetic relationships of FGF-BP3. A.) Alignment of the amino acid sequences for human and mouse FGF-BP3. Regions targeted for peptide antibody production are indicated. B.) Updated radial tree diagram demonstrating species homology of the three known members of the FGF-BP family including the recently identified chicken FGF-BP1

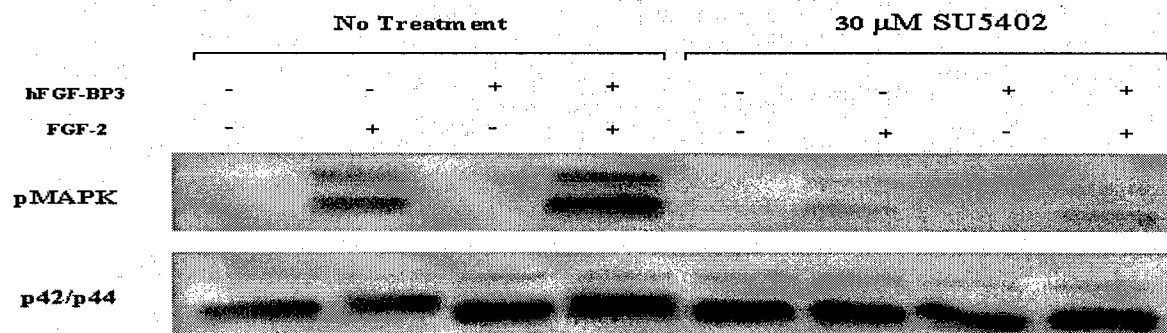


Figure 2: FGF-BP3 enhancement of ERK1/2 phosphorylation ERK1/2 (aka MAPK) phosphorylation and total ERK expression levels were measured in NIH-3T3 cells stimulated with 0.2ng FGF-2 and/or human FGF-BP3 in serum-free conditions via immunoblot analysis. Addition of the FGFR1 inhibitor SU5402 reduced ERK1/2 activation to near baseline levels.

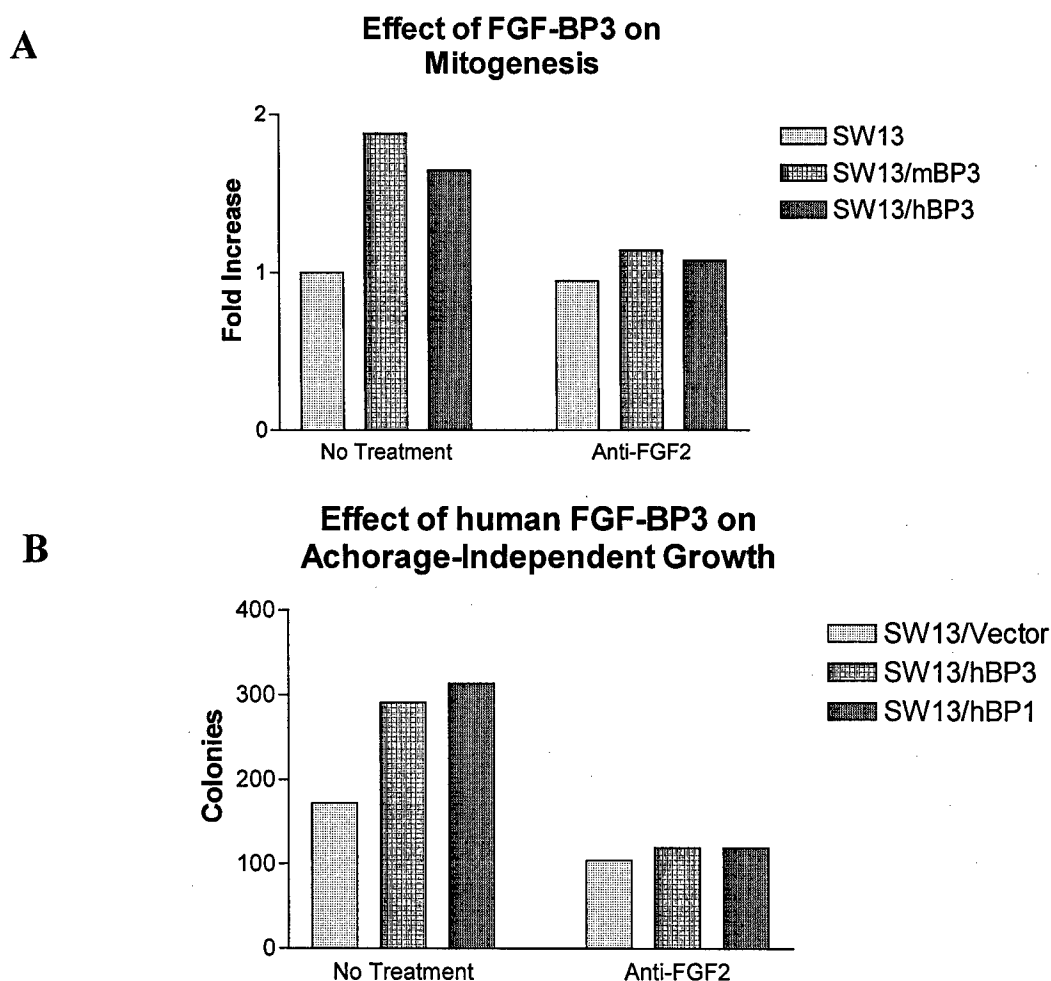


Figure 3: FGF-BP3 modulates SW-13 mitogenesis and anchorage-independent growth. A) WST-1 proliferation measuring 48 hour cell viability of parental SW-13 cells or human and mouse FGF-BP3 expressing SW-13 cells in serum-free conditions in both the absence and presence of 10 μ g/ml of a neutralizing antibody against FGF2 B) Soft agar assay measuring colony formation in both human FGF-BP3 and human FGF-BP1 expressing cells and SW-13 cells

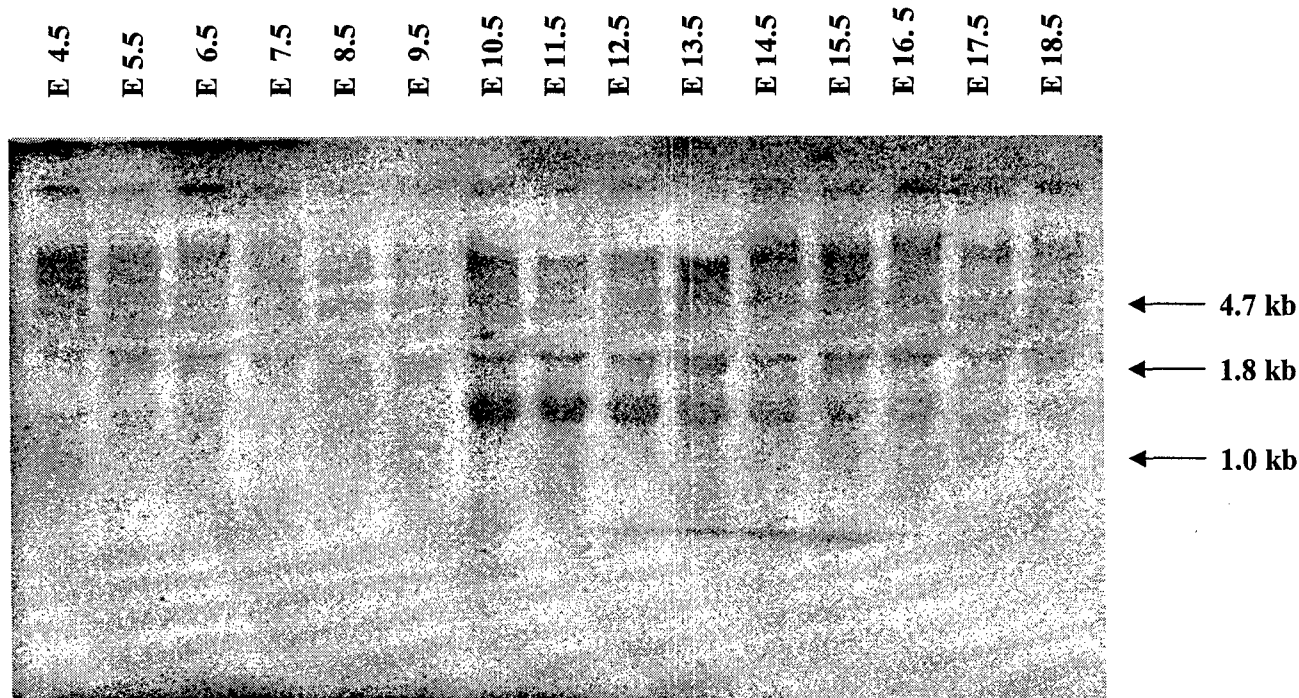


Figure 4: **Northern blot of mouse embryo tissue samples.** A pre-made membrane with samples collected from mouse embryonic tissue from day 4.5 to day 18.5 was probed with a specific mouse FGF-BP3 sequence. As indicated at approximate size of 1.2 kb, a dramatic spike in FGF-BP3 expression was observed at day 10.5.

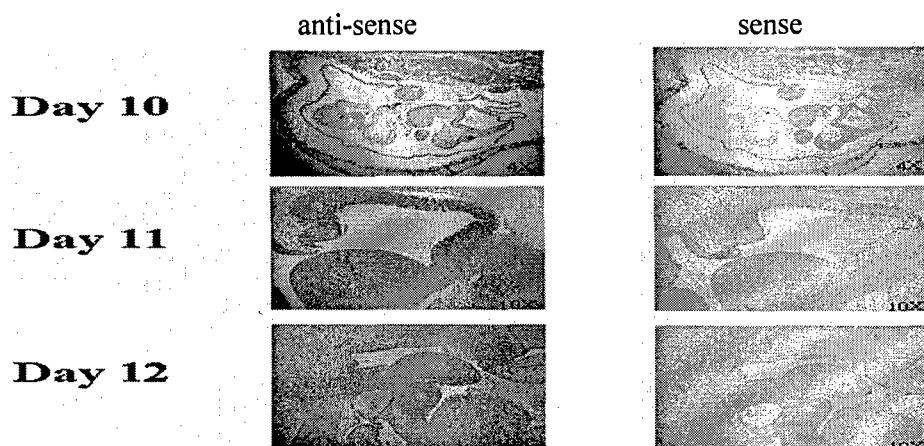


Figure 5: ***In situ* hybridization Analysis of Mouse Embryo Tissue.** Murine FGF-BP3 mRNA expression in a 10- through 12-day old mouse embryo section shows a ubiquitous expression pattern.

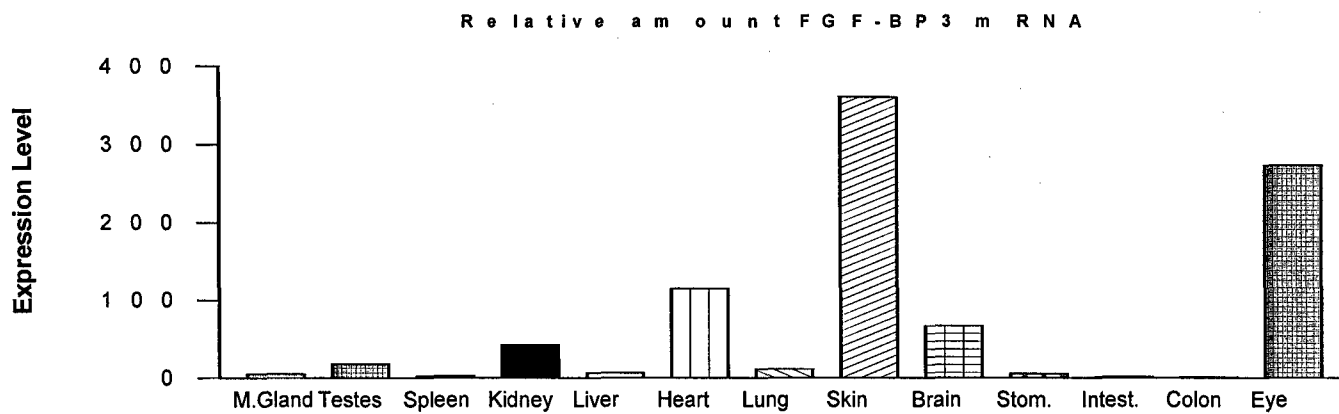


Figure 6: Mouse FGF-BP3 mRNA expression in adult tissue. A sample of 12 organs from four male and four female mice were collected and mRNA harvested for real-time PCR analysis of FGF-BP3 expression. Results were normalized with the mouse Beta-actin gene and graphed in relative amounts based upon a standardized concentration of mouse neuroblastoma cell line N1E-115 FGF-BP3 expression.

Key Research Accomplishments

- Identification of a novel Fibroblast Growth Factor Binding Protein, FGF-BP3
- We have isolated and amplified human and mouse FGF-BP3.
- We have generated vector constructs containing the open reading frame of human and mouse FGF-BP3 and created stable cells lines.
- We have purified recombinant human and mouse FGF-BP3 from cell media.
- We have shown FGF-BP3 can enhance FGF-dependent ERK1/2 phosphorylation, cellular mitogenesis, and anchorage-independent growth in cell lines
- We have demonstrated through cell free binding assays that human and mouse FGF-BP3 bind recombinant FGF2.
- We have observed through Northern Blot analysis a significant increase in mouse mRNA expression in embryonic tissue at day 10.5 with a subsequent decrease in mRNA levels through day 16.5.
- We have generated an in situ hybridization probe for human and murine FGF-BP3 and have confirmed the increase in ubiquitous mouse mRNA expression in embryonic tissue slides beginning at day 10 and observed through day 14.
- We have examined a profile of 12 organs in adult mice to determine relative levels of murine FGF-BP3 levels, finding peak amounts in brain and eye.
- We have generated a peptide antibody that can specifically recognize human FGF-BP3

Conclusions

The goal of our initial project was contingent upon the discover of the murine homologue to human FGF-BP2 and as no such gene could be identified, our direction was altered upon the discovery a novel member of the FGF-BP family in both human and mice, which we chose to call FGF-BP3. We were able to easily isolate the cDNA of each gene and engineer vector constructs allowing us to express the proteins in stable transfected SW-13 cell lines. From these cells, we were able to purify recombinant protein and treat cells with both mouse and human FGF-BP3 in order to determine their biological and biochemical effects. Signal transduction experiments indicated that FGF-BP3 was able to enhance FGF2-dependent ERK1/2 activation. Additionally, we were able to determine that overexpression of both human and murine FGF-BP3 results in an increased level of proliferation and anchorage-independent growth. These results are similar to what has been observed with FGF-BP1 and FGF-BP2 in the same cell lines. As suspected, we have also determined that human and murine FGF-BP3 is bound by FGF2 and attempts to further quantify and characterize this interaction are near completion. Our studies of the expression of FGF-BP3 could not be limited to mouse mammary gland development, as we could not predict the tissue specific profile of FGF-BP3 expression. Our attempts to characterize FGF-BP3 in total mouse development began with Northern blot analysis using a specific murine FGF-BP3 probe which indicated a sizeable increase in mRNA levels in mouse embryonic tissue samples beginning at day 10.5 and diminish through day 16.5. Our data was further justified by *in situ* analysis of mouse embryonic slides that ranged from day 8 through day 16, where a sharp and ubiquitous increase in mRNA staining began around day 10 and decreased around day 14. However, these findings did not achieve our desired result of identifying

target areas of expression, so we surveyed a panel of 12 adult mice organs for relative FGF-BP3 expression and found highest levels to be observed in the brain and eye. Attempts to characterize the role FGF-BP3 expression may play in cancer development in these organs are also near completion. Finally, we have initiated the production of peptide antibodies for specific regions of human and mouse FGF-BP3 as well as FGF-BP2. Final results have identified an antibody specific for human FGF-BP3 but only of use in Immunoblot analysis. This data was presented at the 2005 Era of Hope meeting and we are currently preparing a final manuscript for potential publication.

Abbreviations

FGF: fibroblast growth factor
FGF-BP: fibroblast growth factor binding protein
FGFR1: fibroblast growth factor receptor 1
mBP3: mouse FGF-BP3
hBP3: human FGF-BP3
SCC: squamous cell carcinoma
EST: expressed sequence tag
MAPK: mitogen activated protein kinase
ERK: extracellular signal regulated kinase
IMAC: immobilized-metal affinity chromatography

References

1. Folkman J. & Klagsbrun M. (1987) Angiogenic factors. *Science*, **235**, 442.
2. Folkman J. (1986) How is blood vessel growth regulated in normal and neoplastic tissue? G.H.A. Clowes memorial Award lecture. *Cancer Res*, **46**, 467.
3. Baird A. & Klagsbrun M. (1991) The fibroblast growth factor family. *Cancer Cells*, **3**, 239.
4. Wu D.Q., Kan M.K., Sato G.H., Okamoto T. & Sato J.D. (1991) Characterization and molecular cloning of a putative binding protein for heparin-binding growth factors. *J Biol Chem*, **266**, 16778.
5. Powers C.J., McLeskey S.W. & Wellstein A. (2000) Fibroblast growth factors, their receptors and signaling. *Endocr Relat Cancer*, **7**, 165.
6. Czubayko F., Smith R.V., Chung H.C. & Wellstein A. (1994) Tumor growth and angiogenesis induced by a secreted binding protein for fibroblast growth factors. *J Biol Chem*, **269**, 28243.
7. Czubayko F., Liaudet-Coopman E.D., Aigner A., Tuveson A.T., Berchem G.J. & Wellstein A. (1997) A secreted FGF-binding protein can serve as the angiogenic switch in human cancer. *Nat Med*, **3**, 1137.